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Collagen Biosynthesis in Gastric Cancer: Immunohistochemical Analysis of Prolyl 4-Hydroxylase

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Background and Objectives: The mechanism of the desmoplastic response in gastric carcinoma tissues is largely unknown. The objective of this study is to determine the localization of prolyl 4-hydroxylase (PH), an enzyme that plays a crucial role in collagen biosynthesis.

Methods: Freshly prepared gastric carcinoma tissues from 51 cases, including 13 of the scirrhous type (diffusely infiltrative type), were immunostained by using monoclonal antibodies to human placental PH.

Results: Although cytoplasmic staining for PH was observed in both fibroblasts and carcinoma cells, there was increased expression of the α-subunit in fibroblasts and no difference in expression between the scirrhous and non-scirrhous type gastric carcinomas. In scirrhous type samples, there was increased PH expression in fibroblasts located in the tumor periphery when compared with fibroblasts in the tumor center. These findings suggested that maintenance of a balance between production and degradation of collagen in gastric carcinoma tissues might be important for stroma formation.

Conclusions: It is speculated that activated fibroblasts participate in collagen biosynthesis at the tumor periphery rather than in the tumor center and that increased collagen biosynthesis at the tumor periphery in scirrhous gastric carcinoma may assist further invasion of tumor cells.

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KEY WORDS: **extracellular matrix; desmoplasia; scirrhous carcinoma; cell-cell interaction**

INTRODUCTION

Tumor stroma is composed of tumor cells, extracellular matrix (ECM) proteins, inflammatory cells, and (myo)fibroblasts [1]. It has been shown that the tumor can modify the ECM in the following ways: (1) localized degradation of matrix components associated with invasion, (2) stimulated accumulation of matrix components by host cells in response to the presence of the tumor, known as desmoplasia, and (3) synthesis of matrix components such as collagens by tumor cells [2]. Diffusely infiltrative gastric carcinomas known as scirrhous carcinomas have an abundant deposit of stroma that is particularly rich in collagen, i.e., they exhibit desmoplasia [3–5]. In contrast, intestinal type gastric carcinomas usually have a poor deposit of stroma.

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Scirrhous gastric carcinomas and intestinal type carcinomas differ in their clinical characteristics. Intestinal type gastric carcinomas tend to metastasize to distant organs through the blood circulation, whereas diffuse type gastric carcinomas, particularly scirrhous type carcinomas, tend to metastasize through the lymphatics and disseminate into the peritoneal cavity. Scirrhous type gastric carcinoma has a very poor prognosis.

Recent studies have shown that altered ECM macromolecules can influence not only host cell functions [6,7] but also various tumor cell functions such as morphology [8,9], growth [10], differentiation, and metastatic potential [11–13]. Collagen is the major constituent of the ECM component [14] and several types of collagens have been identified in gastric carcinoma tissues [15–21], but the fibrotic mechanism and the role of collagen in cancer invasion are still controversial.

Prolyl 4-hydroxylase (PH) catalyzes the formation of 4-hydroxyproline in collagens and other proteins with collagen-like amino acid sequences by the hydroxylation of proline residues in peptide linkages [22–24]. The enzyme plays a crucial role as a rate-limiting enzyme in collagen biosynthesis, as the hydroxyl groups of the 4-hydroxyproline residues are essential for the folding of the newly synthesized procollagen polypeptide chains into a triple-helical structure [23,24].

The active PH enzyme is a tetramer of 240 kDa with the subunit structure $\alpha_2\beta_2$ and consists of two different types of inactive monomer with molecular weights of about 64 kDa (α-subunit) and 60 kDa (β-subunit) [25]. The enzyme is located within the rough endoplasmic reticulum, as shown by immunoelectron microscopy [26]. In general, the activity of this enzyme is always elevated in tissues that are actively synthesizing collagen [27]. Increased tissue levels of prolyl hydroxylase activities have been reported in many diseases associated with fibrosis such as hepatic fibrosis [28], wound healing [29], hypertensive vascular lesions [22,30], and carcinomas [31–34]. By using this enzyme as a marker, we can evaluate the synthesis of all types of collagen. The immunohistochemical localization of PH was determined to clarify the mechanisms of collagen biosynthesis in gastric carcinoma and to compare the mechanisms in scirrhous and non-scirrhous gastric carcinomas.

MATERIALS AND METHODS

Cell Lines

The following cell lines were used to test the immunoreactivity in vitro. The fibroblast cell line MY-44 derived from adult human skin was established originally. Three gastric cancer cell lines, MKN-7, 74 [35] and KATO-III [36], were purchased from the IBL CELL BANK (Tokyo, Japan). Each cell line was cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum. For immunocytochemical staining, the cells cultured on Lab-Tek slides (Miles Laboratories, Naperville, IL) for 24 hr were washed three times with Tris-buffered saline (50 mM Tris/HCl, 0.15 M NaCl, pH 7.6) for 5 min and fixed with absolute acetone for 5 min at 4°C.

Tissue Samples

Samples of gastric carcinomas were obtained from 51 cases of surgical specimens resected at the Department of Surgery, School of Medicine, Keio University, and the Department of Surgery, National Tokyo Medical Center. There were 11 cases of t1 carcinomas and 40 cases of more advanced carcinomas (>t2). Histologically, there were 21 intestinal type carcinomas and 30 diffuse type carcinomas. These included 13 diffusely infiltrative (scirrhous) carcinomas.

Fresh surgical specimens were embedded in Tissue Tek O.C.T. Compound (Miles Laboratories) and were rapidly frozen within 30 min after resection. In each case, several samples were taken from the macroscopic lesions as well as the surrounding intact areas. Cryostat sections 4 μm in thickness were fixed with absolute acetone for 5 min at 4°C before staining.

Antibodies

Mouse monoclonal antibodies raised against the α- and β-subunits of human placental PH were purchased from Fuji Chemical Industries (Kanazawa, Japan). The specificity of the antisera has been previously shown [37].

Immunohistochemistry

Immunostaining was performed according to the avidin-biotin-peroxidase complex (ABC) method [38] with slight modifications. Sections were treated with 0.6% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity. After immersion in Tris-buffered saline (TBS), pH 7.6, for 5 min, the sections were incubated for 20 min with normal rabbit serum (DAKO Corporation, Carpinteria, CA) diluted 1:10 in TBS to eliminate nonspecific staining. After removal of excess normal serum, incubation was carried out for 30 min with primary antibodies diluted 1:50 in TBS. After treatment for 30 min with biotinylated anti-mouse rabbit antibody (DAKO) diluted 1:400 in TBS, ABC (DAKO) was applied for 30 min. Between each step, sections were washed three times in TBS for 15 min each. Immunoreactivity for each subunit was detected by treatment with 0.1% 3,3′ diaminobenzidinetetrahydrochloride (Sigma, St. Louis, MO) and 0.003% hydrogen peroxidase in TBS for 5 min. The sections were counterstained with Mayer's hematoxylin for 10 min, then dehydrated in ethanol, rinsed in xylene, and mounted with coverslips. Control sections were treated with non-immune mouse serum (DAKO) instead of the primary antibodies at an equiva-

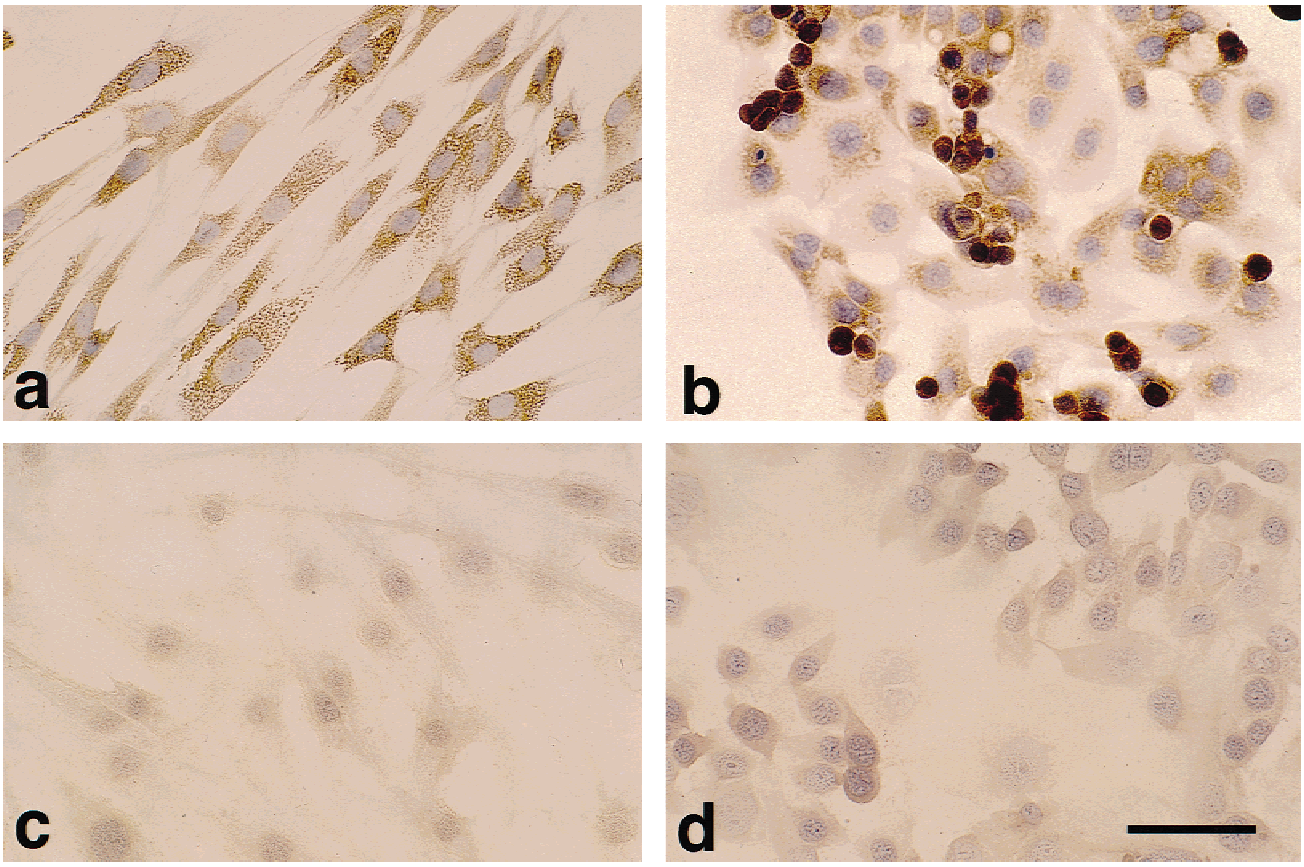

Fig. 1. Immunolocalization of PH (β-subunit) in cell lines. Cytoplasmic staining of enzyme is observed in both **(a)** human skin fibroblasts (MY-44) and **(b)** a gastric carcinoma cell line (MKN-7). Immunostaining with nonimmune mouse IgG as a negative control for **(c)** MY-44 and **(d)** MKN-7. Hematoxylin counterstain. Bar = 50 μm.

lent concentration of IgG. All reactions were performed at room temperature.

Assessment of Results

If more than 50% of cells in one representative section of the tumors clearly showed cytoplasmic staining, these cases were classed as positive. The overall PH-positive cases were defined as those cases in which cancer cells and/or fibroblasts were positively stained. In addition to an overall assessment, the pattern of PH immunostaining in the tumor center was compared with that at the tumor periphery. The tumor periphery was defined as either the deep margin or the invading edge of the tumor tissues. Routine hematoxylin-eosin (H&E) staining was also performed for conventional evaluation. In some cases of diffuse type adenocarcinomas, PAS staining was performed to distinguish inflammatory cells from cancer cells using serial sections.

Statistical Analysis

Gastric carcinomas were divided into either scirrhous type or non-scirrhous type according to the amount of stroma. Comparison of the PH-positive rate (positive cases/total cases) of cancer cells and fibroblasts at the tumor center and the tumor periphery was done using the chi-squared test. Two values were considered significantly different when $P < 0.05$ and suggestively different when $P < 0.10$.

RESULTS

Staining Pattern of PH in Cell Lines

PH expression was observed in the cytoplasm of both fibroblasts and cancer cells. Representative result is shown in Figure 1. The immunoreactivity of the β-subunit was more intense than that of the α-subunit (data not shown).

Staining Pattern of PH in Gastric Carcinoma Tissues

In normal gastric tissue, there was no immunoreactivity for the α-subunit. β-Subunit-positive glandular cells, particularly chief cells, were observed in the mucosal layer. Other mesenchymal cells such as fibroblasts or inflammatory cells were negative for PH (data not shown).

In carcinoma tissues, cytoplasmic staining for PH was

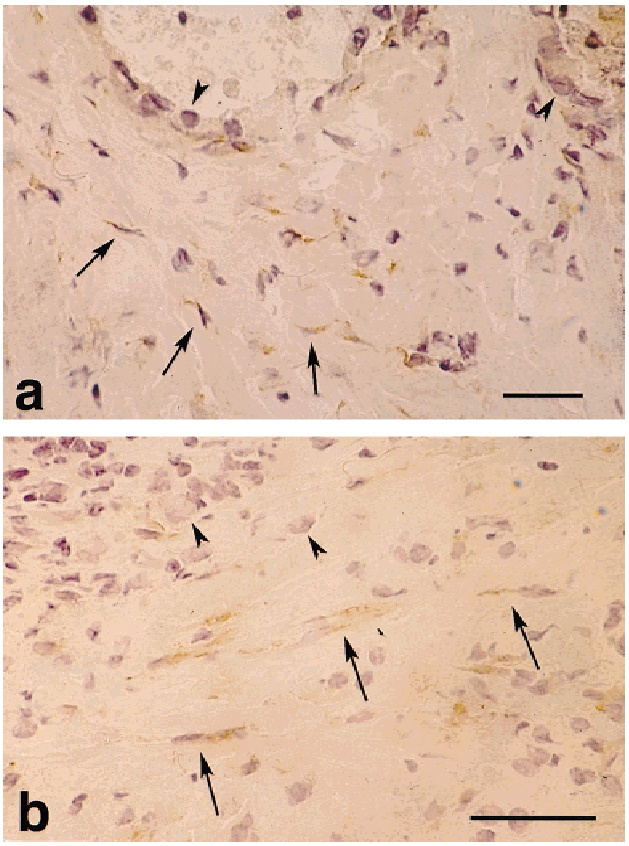

Fig. 2. Immunostaining for PH (α-subunit) in gastric carcinoma tissues. Sections of **(a)** intestinal type and **(b)** diffusely infiltrative (scirrhous) type gastric carcinoma tissues with abundant stroma immunostained with anti-α-subunit. In both sections, only fibroblasts, indicated by arrows, are stained. Cancer cells (arrowheads) are not stained. Bars = 50 μm.

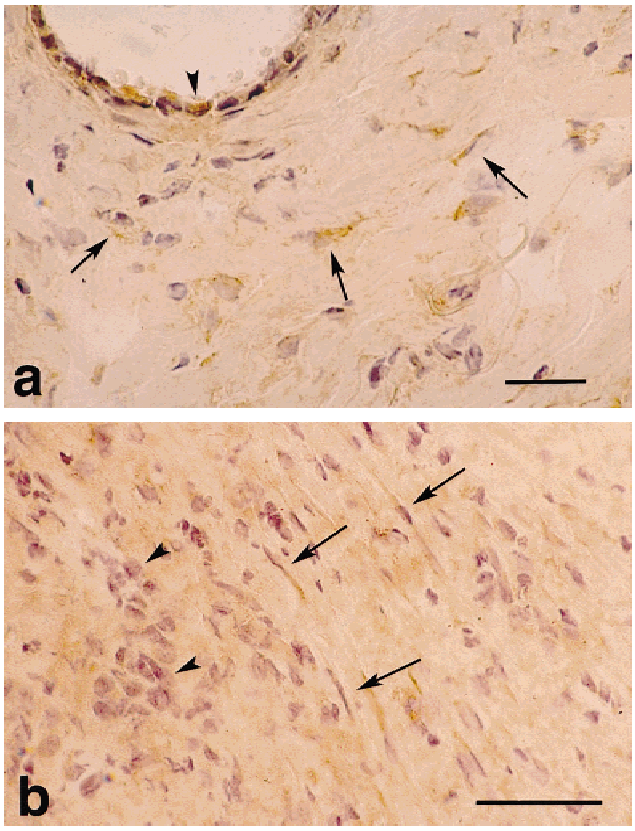

Fig. 3. Immunostaining for PH (β-subunit) in gastric carcinoma tissues. Serial sections of Figure 2 immunostained with anti-β-subunit. Both cancer cells (arrowheads) and fibroblasts (arrows) are stained in **(a)** intestinal type and **(b)** diffusely infiltrative (scirrhous) type gastric carcinoma tissues. Bars = 50 μm.

observed in fibroblasts and/or cancer cells (Figs. 2, 3). The positive rate for the α-subunit in cancer cells was 4/46 (8.7%) and 22/46 (47.8%) in fibroblasts. Overall positivity was 23/46 (50%), and in 3 cases (6.5%) both types of cells were simultaneously positive. The positive rate for β-subunit in cancer cells was 48/51 (94.1%) and 35/51 (68.6%) in fibroblasts. Overall positivity was 50/51 (98.0%) and both types of cells were positive for staining in 33 cases (64.7%). The α-subunit-positive cells were always β-subunit-positive.

Localization of PH-Positive Cells in Gastric Carcinoma Tissues

The overall PH-positive rate (positive cases/total cases) in diffusely infiltrative (scirrhous) type gastric carcinomas according to the α- and β-subunits was 7/9 (77.8%) and 13/13 (100%), respectively. In non-scirrhous type (intestinal type gastric carcinomas and diffuse type gastric carcinomas without abundant stroma), the PH-positive rate for each subunit was 16/37 (43.2%) and 37/38 (97.4%), respectively. The difference in expression of the α-subunit between scirrhous and non-scirrhous carcinomas was suggestively different ($P = 0.063$).

The PH-positive rate in fibroblasts and cancer cells was compared at the tumor center and the tumor periphery (Figs. 4–7). In scirrhous type gastric carcinomas, the α-subunit-positive rate for fibroblasts at the tumor center was 2/9 (22.2%) and at the tumor periphery was 6/9 (66.7%). The α-subunit-positive rate tended to be higher at the tumor periphery compared to the tumor center ($P = 0.058$). The β-subunit-positive rate for fibroblasts at the tumor center was 6/13 (46.2%) and at the tumor periphery was 11/13 (84.6%). The positive rate was significantly higher at the tumor periphery ($P = 0.039$). In non-scirrhous type carcinomas, the α-subunit-positive rate for fibroblasts at the tumor center was 11/37 (29.7%) and at the tumor periphery was 16/37 (43.2%). The β-subunit-positive rate for fibroblasts at the tumor center was 21/38 (55.3%) and at the tumor periphery was 24/38 (63.2%). There were no significant differences in the positive rate between these regions in both subunits (Fig. 6).

On the other hand, the α-subunit-positive rate for cancer cells in scirrhous type gastric carcinomas was 2/9 (22.2%) at the tumor center and 1/9 (11.1%) at the tumor

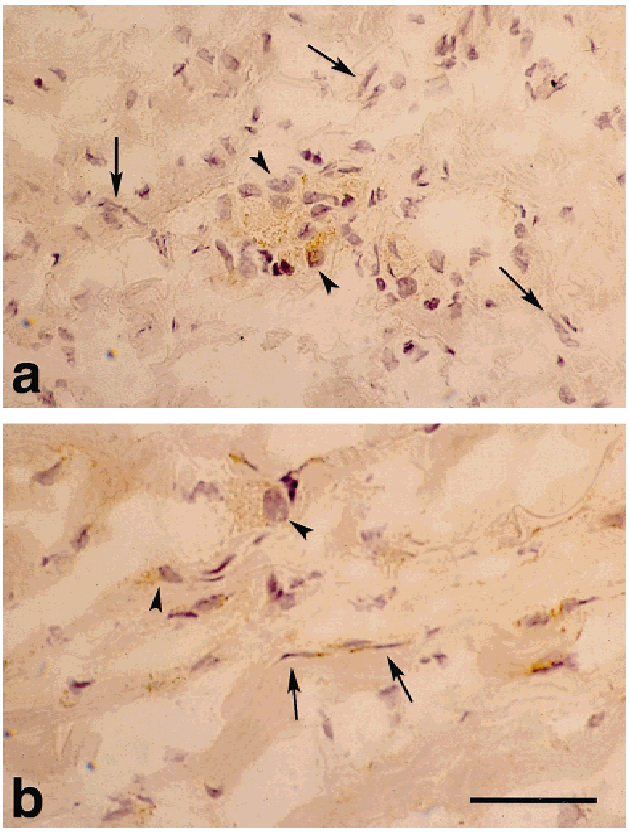

Fig. 4. Comparison of the PH (β-subunit) staining pattern between the tumor center and periphery in diffusely infiltrative (scirrhous) type gastric carcinoma. Sections of diffusely infiltrative (scirrhous) type gastric carcinoma tissue with abundant stroma immunostained with anti-β-subunit. **(a)** Only cancer cells (arrowheads) are stained in the tumor center. Fibroblasts (arrows) are not stained. **(b)** In contrast, both cancer cells (arrowheads) and fibroblasts (arrows) are stained at the tumor periphery. Bar = 50 μm.

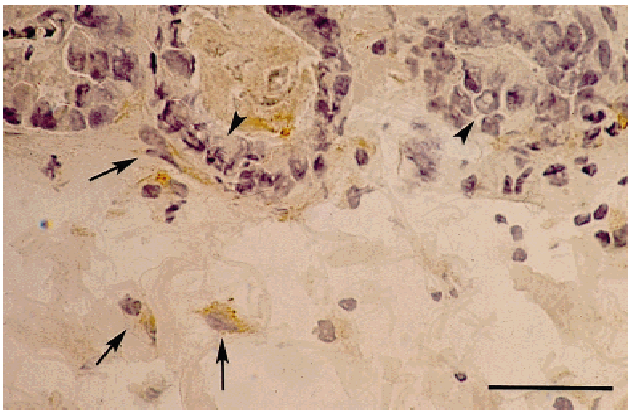

Fig. 5. Section of intestinal type gastric carcinoma tissue immunostained with anti-α-subunit. Only fibroblasts (arrows) are stained at the tumor periphery, as in the tumor center shown in Figure 2a. Cancer cells (arrowheads) are not stained. Bar = 50 μm.

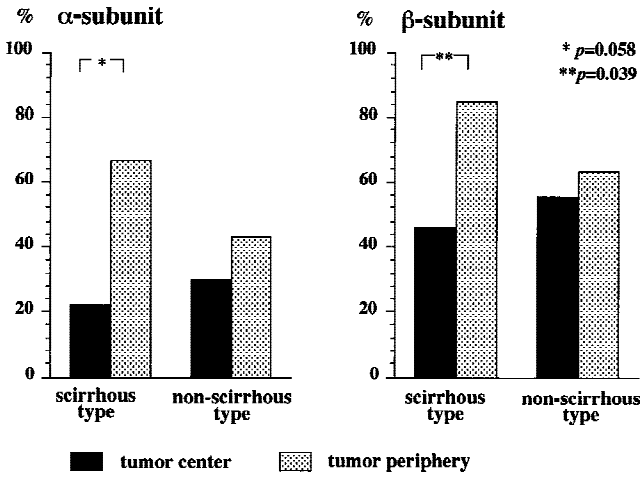

Fig. 6. PH-positive rate (positive cases/total cases) in fibroblasts. In diffusely infiltrative (scirrhous) type gastric carcinomas, the α-subunit-positive rate tended to be higher at the tumor periphery than in the tumor center ($P = 0.058$). The β-subunit-positive rate was significantly higher at the tumor periphery ($P = 0.039$). However, in non-scirrhous type carcinomas (intestinal type carcinomas and diffuse type carcinomas without abundant stroma), there were no significant differences in levels of subunit expression between the regions.

periphery. In non-scirrhous type gastric carcinomas, the positive rate was 2/37 (5.4%) at both regions. The β-subunit-positive rate for cancer cells in scirrhous type gastric carcinomas was 13/13 (100%) at the tumor center and 11/13 (84.6%) at the tumor periphery. In non-scirrhous carcinomas, the positive rate at the tumor center was 35/38 (92.1%) and at the tumor periphery was 33/38 (86.8%). The PH-positive rate for cancer cells was not significantly different between these regions (Fig. 7).

DISCUSSION

In the present study, the overall positive rate for the α-subunit was 50% with a higher level of expression seen in fibroblasts. The overall positive rate for β-subunit expression was 98% and carcinoma cells and/or fibroblasts were positive for this subunit. All cells that expressed the α-subunit in carcinoma tissues also expressed the β-subunit. The inactive form of the β-subunit is produced in an excess to the α-subunit and forms a β-subunit pool [24]. Assembly of active enzyme requires the formation of tetramers by newly synthesized α-subunits and preformed β-subunits [25]. The α-subunit contributes the major part of the catalytic site of the enzyme [24], therefore, our results indicate that fibroblasts play an important role in the production of collagens in gastric carcinoma tissues.

Several studies have disclosed that carcinoma cells [18,28,39,40] or host (myo)fibroblasts [3,16,19,20,21,41–43] play important roles in the production of collagens. Recent immunoelectron microscopic studies using monoclonal antibody [19,21] and in situ hybridization studies of type I collagen mRNA expression [41] indicated that host (myo)fibroblasts synthesize type I collagen in gastrointestinal carcinoma tissues. An in situ hybridization study

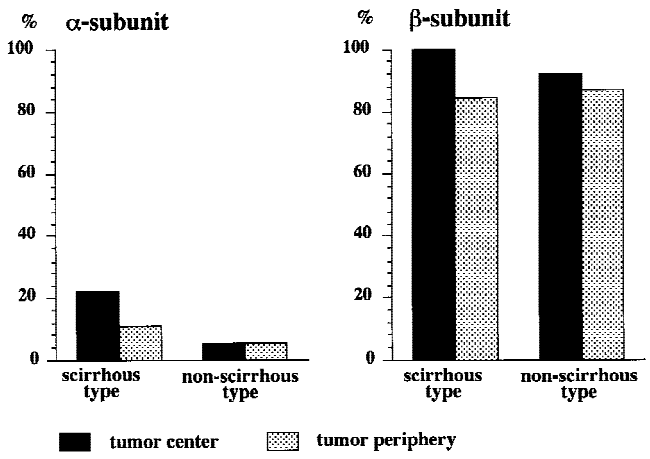

Fig. 7. PH-positive rate (positive cases/total cases) in carcinoma cells. In both scirrhous and non-scirrhous type gastric carcinomas, the α-subunit-positive rate was low and the β-subunit-positive rate was high at the tumor center and tumor periphery. There was no significant difference in subunit expression between the regions.

by Hewitt et al. [44] on colorectal carcinomas indicated that type IV collagen mRNA is expressed in vascular endothelial cells in superficial tumor areas and the tumor center.

Al-Adnani et al. [39] reported that immunohistochemical localization of prolyl hydroxylase was observed specifically in breast carcinoma cells and not in stromal cells. They concluded that carcinoma cells actively synthesize collagen in breast carcinoma tissues. In this current study of gastric carcinoma tissues, the collagen biosynthesis mechanism in gastric carcinoma tissue may differ from that in breast carcinoma tissues.

Recent studies have reported that several factors secreted by carcinoma cells influence the production of collagen from host fibroblasts. These factors include transforming growth factor-β (TGF-β) in scirrhous gastric carcinoma [45], and a 68 kDa polypeptide [46] and collagen stimulating factor (COSF) [7] in breast carcinoma. In carcinoma tissues, collagen biosynthesis from fibroblasts is elevated, in part, by these factors.

There was no significant difference in the expression patterns of the α- and β-subunits between scirrhous type (diffusely infiltrative type) and non-scirrhous type (intestinal type and diffuse type without abundant stroma) gastric carcinoma tissues. In previous studies, we have reported that the tissue homogenate of both scirrhous and non-scirrhous gastric carcinomas showed elevated prolyl hydroxylase activity [34]. Types I and III collagen mRNA expression was higher in gastric carcinoma tissues compared with the normal tissue [45], and diffuse type gastric carcinoma tissues had fewer fibroblasts positive for type I collagen mRNA than intestinal type carcinomas [41]. These findings indicated that collagen biosynthesis is increased in both types of gastric carcinoma, irrespective of the amount of stroma, and that simple overproduction of collagen does not explain the net result of stroma formation. Therefore, regulation of stroma formation may be better understood by evaluation of the balance between the production and the degradation of matrix proteins.

Degradation of matrix proteins such as collagen is a crucial step in the invasion of the surrounding tissues by tumor cells. A growing number of reports have implicated the matrix metalloproteinases (MMPs) such as interstitial collagenase and gelatinases in cancer cell invasion and metastasis [2,47,48]. Several correlative studies have suggested that MMPs might be responsible for the malignant features of cancer. Kubochi et al. [49] showed high levels of collagenase activity at the invading edge of gastric carcinomas. In colorectal carcinomas, van der Stappen et al. [50] found that raised collagenase activity was associated with deeper invasion and poorer tumor differentiation, and Hewitt et al. [51] showed immunohistochemical localization of collagenase in the connective tissue of stroma of colorectal carcinomas and an increase in staining intensity close to the invasive edge. Recent in situ hybridization studies also showed expression of interstitial collagenase mRNA in the stroma of colonic adenocarcinomas [52]. These findings suggested that collagen degradation is increased in carcinoma tissues, particularly at the invading edge where the interaction between carcinoma cells and host cells such as fibroblasts and inflammatory cells might play an important role. Actual deposition of tumor stroma can thus be attributed to an imbalance between production and degradation of stroma.

The present study is the first report to show that the positive rate for the α-subunit in scirrhous gastric carcinomas at the tumor periphery tended to be high and that the β-subunit was significantly more highly expressed at the tumor periphery than in the tumor center. However, in non-scirrhous carcinomas, there were no significant differences between these regions. These results suggested that, in the scirrhous type, collagen biosynthesis in fibroblasts may be greater at the tumor periphery than in the tumor center, the desmoplastic response taking place at the tumor periphery. Takeuchi [33] showed lower levels of prolyl hydroxylase activity in the fibrous region of scirrhous carcinoma tissues compared with the neighboring edematous region where there were lower amounts of hydroxyproline deposits. Watanabe et al. [21] showed more prominent proliferation of myofibroblasts and deposition of type III collagen in the marginal portion of poorly differentiated gastric carcinoma tissues compared with the central portion.

In the tumor center, accumulated or modified ECM macromolecules can influence various tumor cell and fibroblast functions. Several studies have shown that the growth rate [53] and the collagen synthesis [54] were

suppressed when fibroblasts were cultured in collagen gel.

In scirrhous gastric carcinomas, activated fibroblasts in the tumor periphery participate in the biosynthesis of collagen, and accumulated collagen in turn influences carcinoma cell functions, which leads to tumor invasion. Although desmoplasia has been thought to be a protective phenomenon against tumor invasion [5,55], the response in scirrhous gastric carcinoma might actually benefit the invasion of tumor cells.

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